

1,N²-Propanodeoxyguanosine Adducts: Potential New Biomarkers of Smoking-induced DNA Damage in Human Oral Tissue¹

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Abstract

Highly DNA-reactive α,β -unsaturated aldehydes such as acrolein and crotonaldehyde are common environmental pollutants present in cigarette smoke and automobile exhaust and are also released endogenously by lipid peroxidation. Acrolein- and crotonaldehyde-derived 1,N²-propanodeoxyguanosine (AdG and CdG, respectively) have been detected in the tissues of carcinogen-treated rodents and as background lesions in DNA from humans and untreated rodents. To determine whether cigarette smoking increases the levels of AdG and CdG, gingival tissue DNA from 11 smokers (4 males and 7 females; 30–58 years old) and 12 nonsmokers (8 males and 4 females; 21–66 years old) was analyzed using a previously described ³²P-postlabeling high-performance liquid chromatography method. The results showed that the mean AdG levels in smokers were significantly higher than those in nonsmokers (1.36 ± 0.90 $\mu\text{mol/mol}$ guanine in smokers versus 0.46 ± 0.26 $\mu\text{mol/mol}$ guanine in nonsmokers; $P = 0.003$). The mean CdG 1 levels in smokers and nonsmokers were 0.53 ± 0.44 and 0.06 ± 0.07 $\mu\text{mol/mol}$ guanine, respectively, corresponding to an 8.8-fold increase for smokers ($P = 0.0015$). Similar to CdG 1, levels of CdG 2 were increased 5.5-fold in smokers as compared to nonsmokers, from 0.31 ± 0.40 to 1.72 ± 1.26 $\mu\text{mol/mol}$ guanine ($P = 0.0014$). Furthermore, the total levels of cyclic adduct (AdG and CdG) in smokers were 4.4-fold greater than those in nonsmokers ($P = 0.0003$). This study shows the detection of the potentially promutagenic 1,N²-propanoguanine adducts in human oral tissues and demonstrates for the first time an increase of structurally identified adducts in oral tissue DNA by cigarette smoking.

Introduction

Cigarette smoking has been causally associated with cancers at several sites in humans, including the oral cavity (1, 2). The development of convenient biomarkers for DNA damage caused by cigarette smoking is important in identifying the segment of smokers who are at high risk of developing neoplastic malignancies. Although information about exposure to various tobacco carcinogens may be obtained from analysis of urinary metabolites and/or hemoglobin or protein adducts (3–6), specific genetic biomarkers are perhaps the most direct and relevant to cancer risks. These biomarkers could provide valuable qualitative and quantitative information about structures and potential mutagenic characteristics of the persistent DNA damage resulting from exposure to carcinogens in cigarette smoke. Cigarette smoke contains a number of known carcinogens, including polyaromatic hydrocarbons, nitrosamines, and heterocyclic amines (7). Whereas some studies showed an increase of the nonpolar polycyclic aromatic hydrocarbons-type adducts in DNA obtained from

oral biopsy samples and the exfoliated mucosal cells (8, 9), others failed to show such an increase (10–12). There is, however, a general consensus that DNA isolated from various tissues of smokers, such as the lungs, contains higher levels of polycyclic aromatic hydrocarbons-like adducts as compared to nonsmokers (13–15), but the structures of these DNA modifications have not been identified.

In addition to these carcinogens, cigarette smoke also contains relatively high concentrations of reactive aldehydes. Among them are acrolein and crotonaldehyde, the two simplest α,β -unsaturated aldehydes or enals. Enals are known to modify DNA bases without bioactivation by forming cyclic propano adducts (16, 17). Both acrolein and crotonaldehyde are found in cigarette smoke and automobile exhaust and are produced by burning fatty food (18, 19). AdG adducts have been detected by immunoassay in *Salmonella* tester strains TA 100 and TA 104 on incubation with acrolein under conditions known to induce revertants (20). Moreover, acrolein may initiate bladder cancer in rats under certain conditions (21). AdG was also found in the lymphocyte DNA of a cyclophosphamide-treated dog (22). CdG adducts have been detected in the liver of rats treated with crotonaldehyde or N-nitrosopyrrolidine, a hepatocarcinogen that yields crotonaldehyde on metabolism (23). Crotonaldehyde has been shown to induce liver tumors in rats (24). Consistent with their potential in mutagenesis and carcinogenesis, site-specific mutagenesis studies have shown that cyclic 1,N²-propanoguanine adducts, if present in DNA, are likely to induce mutations (25, 26). Our studies have shown that diastereoisomers of AdG and CdG, shown in Fig. 1, are present in the tissues of humans and untreated rodents as background DNA lesions (27–29). Endogenous enals produced by lipid peroxidation are likely to contribute to their formation, which may perhaps constitute a more important source than environmental enals (28). Because these DNA lesions may play a role in cancer development, it is important to assess them as biomarkers for monitoring DNA damage in humans. To this end, in this study, we used a ³²P-postlabeling-HPLC method developed previously for exocyclic adducts (27, 29) to analyze levels of AdG and CdG in the gingival DNA of smokers and nonsmokers.

Materials and Methods

Samples of oral tissue obtained from surgery at the periodontal clinic of New York University Dental Center were frozen at -80°C . In all cases, gingival tissues were used for this study, except for one case (see Table 1) in which buccal mucosa was used. Table 1 shows the age and sex of the patients and the self-reported number of cigarettes smoked. The tissues (50–300 mg each) from 12 nonsmokers and 11 smokers were thawed, washed with saline minced, suspended in 2 ml of PBS, and incubated with 0.5 mg/ml collagenase (Sigma; Type IV) and 5 mM CaCl_2 for 10 min at 37°C . Tissues were then washed twice with saline, and DNA was extracted using a procedure involving sequential phenol and chloroform/isomyl alcohol extractions and treatment

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²The abbreviations used are: AdG, acrolein-derived 1,N²-propanodeoxyguanosine; CdG, crotonaldehyde-derived 1,N²-propanodeoxyguanosine; GSH, reduced glutathione; HPLC, high-performance liquid chromatography.

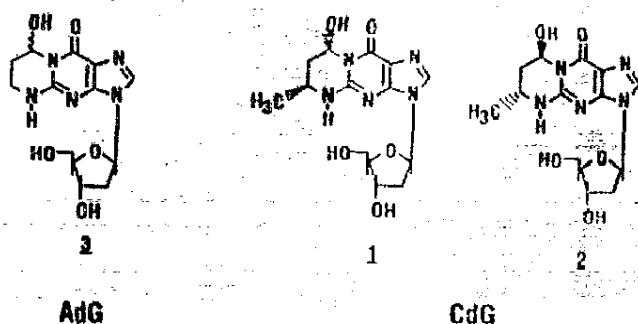


Fig. 1. Structures of isomers of AdG and CdG detected *in vivo*. AdG 1 and AdG 2 are not shown. The diastereomers of CdG 1 and CdG 2 are arbitrarily assigned.

Table 1. AdG and CdG levels in the gingival DNA of nonsmokers and smokers

	Age (yr)	Sex	Cigarettes/day	pmol/mol guanine		
				AdG 3	CdG 1	CdG 2
Nonsmokers						
1	45	M		0.424	0.026	0.038
2	43	F		0.409	0.039	0.048
3	41	M		0.404	0.040	0.101
4	33	M		0.335	0.033	0.119
5	27	F		0.409	0.104	0.216
6	21	F		0.747	0.108	0.351
7	38	M		0.321	0.030	0.071
8	47	F		0.410	0.026	0.177
9	65	M		0.328	0.031	0.210
10	45	M		0.237	0.041	0.151
11	66	M		0.287	ND ^c	1.337
12	31	M		1.167	0.262	0.891
Smokers						
1 ^a	58	M	NA ^b	0.644	0.242	0.591
2	39	F	15	0.977	0.331	1.024
3	43	F	10-15	1.243	0.633	1.444
4	43	F	10	1.022	0.319	0.749
5	30	M	5-7	1.590	1.692	4.334
6	42	F	5-10	2.488	0.924	3.935
7	55	M	NA	1.030	0.372	1.270
8	51	M	20	1.202	0.559	2.118
9	51	F	10-12	0.808	0.335	1.149
10	45	F	10-15	0.517	0.329	0.913
11	36	F	12	3.588	0.119	1.359

^a Buccal mucosal DNA was used.

^b NA, not available.

^c ND, not detected.

with ribonuclease A, T1, and protease (30). Typical DNA yields were ~1 µg/mg tissue, and the purity was checked by the ratios of absorbance at 260/280 (1.8-2.0). The procedures used for detection and quantification of AdG and CdG in DNA were described previously (27, 28). For purification and analysis by reverse-phase HPLC, C18 ODS-3 columns (4.5 × 250 mm; Phenomenex, Torrance, CA) were used. Briefly, 23-122 µg of DNA were enzymatically hydrolyzed and analyzed by HPLC, and the fractions with retention times corresponding to those of AdG- and CdG 3'-monophosphates were collected. Portions of DNA digest (equivalent to ~10 µg of DNA) from each sample were used to quantify the amount of DNA and to determine the efficiency of enzyme hydrolysis. AdG and CdG fractions were treated with nuclease P1 and ³²P-postlabeled in the presence of T4 polynucleotide kinase. The labeled digests were purified first by one-dimensional TLC followed by sequential reverse-phase and ion-pair HPLC analysis. The purified adduct bisphosphates were finally analyzed by reverse-phase HPLC with detection by a radioflow detector, and adduct levels were quantified from radioactivity in the peaks by adjusting for the recovery of simultaneously labeled AdG and CdG as external standards. For confirmation of the identity of the adducts, portions of each purified AdG and CdG adduct bisphosphate from individual samples in each group were pooled into one sample and enzymatically converted to the corresponding 5'-monophosphates. The comigration of labeled adduct 5'-monophosphates obtained with the authentic UV standards was considered confirmation of identity of the *in vivo* adducts (27, 29).

Results and Discussion

Fig. 2 shows typical HPLC chromatograms of comigration of the radioactive peaks obtained from a nonsmoker's and a smoker's DNA with the authentic UV standards of AdG 3, CdG 1, and CdG 2. These results showed, for the first time, the presence of these exocyclic adducts in human oral tissue DNA. Of the three isomers of AdG, AdG 3 was the predominant isomer detected in oral tissue DNA. This observation was consistent with previous results obtained from analysis of other human tissues (27, 29). Table 1 shows the levels of AdG 3, CdG 1, and CdG 2 of each subject from both smokers and nonsmokers. The significant variations for the background adduct levels in nonsmokers are likely caused by individual variability, although the intrinsic variability of the method may also contribute to the differences observed. Other reasons for the background variability could include factors known to influence lipid peroxidation, such as alcohol consumption, dietary fat, intake of antioxidant supplements, and nutritional factors. Unfortunately, lifestyle information other than smoking status was not available. In smokers, we did not see a positive correlation between the number of cigarettes smoked and the adduct levels. A number of reasons, such as the small sample size, inaccurate self-reported number of cigarettes smoked, and assay variability, could account for the lack of correlation. Of course, individual differences in detoxification and repair may be also important.

In Fig. 3, the mean levels of AdG 3, CdG 1, CdG 2, and total adducts (AdG and CdG) were compared between smokers and nonsmokers. The AdG level in smokers and nonsmokers was 1.36 ± 0.90

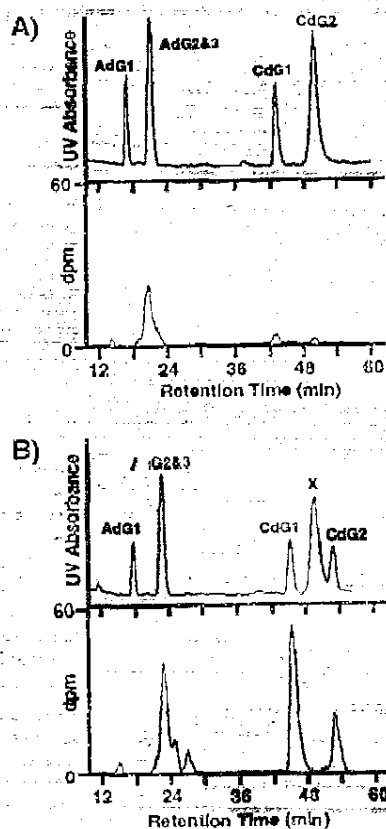


Fig. 2. Typical HPLC chromatograms showing the comigration of purified radioactive peaks obtained from (A) a nonsmoker's DNA (63 µg) and (B) a smoker's DNA (39.8 µg) with the synthetic UV standards. The UV peak marked X in B is an unidentified compound present in some samples.

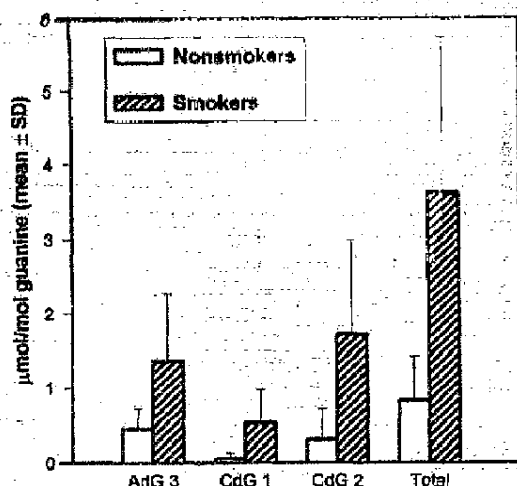


Fig. 3. Comparison of the mean levels of cyclic DNA adducts [AdG 3 ($P = 0.003$), CdG 1 ($P = 0.0015$), CdG 2 ($P = 0.0014$) and total adduct levels ($P = 0.0003$)] in oral tissue between smokers and nonsmokers.

and 0.46 ± 0.26 $\mu\text{mol/mol}$ guanine, respectively, corresponding to a 3.0-fold increase in smokers ($P = 0.003$). The CdG 1 level in smokers was 0.53 ± 0.44 $\mu\text{mol/mol}$ guanine, equivalent to an 8.8-fold increase from 0.06 ± 0.07 $\mu\text{mol/mol}$ guanine found in nonsmokers ($P = 0.0015$). The increase in CdG 2 level from 0.31 ± 0.40 $\mu\text{mol/mol}$ guanine in nonsmokers to 1.72 ± 1.26 $\mu\text{mol/mol}$ guanine in smokers indicates a 5.5-fold increase ($P = 0.0014$). When the total adduct levels were examined, smokers showed a 4.4-fold increase as compared with nonsmokers. These results showed that AdG and CdG levels in oral tissues were significantly elevated by smoking, and the increases in CdG levels seemed to be greater than those of AdG 3. Comparable increases in smokers' adduct levels were also seen after the enzymatic conversion to the corresponding 5'-monophosphates in the confirmation experiments.

The background levels of AdG and CdG found in the gingival tissues of nonsmokers are believed to be mostly derived from the acrolein and crotonaldehyde released by endogenous oxidation of membrane fatty acids and, to a lesser extent, from environmental sources (28). The increased AdG and CdG levels in smokers' gingival DNA could result from direct exposure to acrolein and crotonaldehyde in the cigarette smoke and/or an increased production of endogenous enals via stimulation of lipid peroxidation by the oxidants in the cigarette smoke. The possibility that the increase is due to exposure to the aldehydes in smoke is supported by the fact that enals come in direct contact with oral tissue during smoking, and they do not need bioactivation for the formation of AdG and CdG (16). However, this does not explain the greater increases in CdG than in AdG, because the concentration of acrolein (3–220 $\mu\text{g/cigarette}$) in cigarette smoke is comparable, if not higher, than that of crotonaldehyde (10–228 $\mu\text{g/cigarette}$; Refs. 18 and 19), and acrolein is more reactive toward DNA than crotonaldehyde (16). It is tempting to speculate that the greater increase of CdG could be due in part to the presence of 1,3-butadiene (16–70 $\mu\text{g/cigarette}$) and *N*-nitrosopyrrolidine (30–390 $\mu\text{g/cigarette}$) in cigarette smoke (31, 32). Both are known to be metabolized to crotonaldehyde (33, 34). Although the metabolism of these compounds by oral tissue has not been reported, it is conceivable that crotonaldehyde as a metabolite could conjugate with GSH, which is then transported to the gingival tissue via circulation.

An alternate route of exposure to enals is through their generation by stimulated lipid peroxidation in smokers. The pro-oxidant state in smokers has been previously characterized by the higher plasma

levels of malondialdehyde, the lower GSH levels, and increased 8-hydroxydeoxyguanine levels in leukocyte, oral mucosa, and lung DNA (35–38). The plasma GSH levels return to normal on smoking cessation (39). The depletion of hepatic GSH by treatment with L-buthionine-(S,R)-sulfoximine caused a dramatic increase in the 1,*N*²-propanodeoxyguanosine adduct levels in rat liver DNA (40). Interestingly, in that study, the increases of CdG in the L-buthionine-(S,R)-sulfoximine-treated rats were much more pronounced than those in AdG, a result similar to that obtained from the present study in smokers. These observations seem to lend support to the possibility that endogenous pathways may play a major role in smoking-induced AdG and CdG levels. Regardless of the mechanism(s), this study is the first report of structurally identified DNA adducts in human oral tissue whose levels were significantly increased by smoking. In view of the promutagenic potential of these adducts, the detection of elevated levels of AdG and CdG in oral tissue DNA of smokers may provide a tool for monitoring the risk of smoking-induced oral cancer in humans.

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